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Mutation in polymerase gamma (POLG) have led to depletion of mitochondrial DNA (mtDNA) and mutations in mtDNA. This proposal seeks to determine the effect of POLG mutations on tumorigenesis of breast cancer by altering mtDNA. A mutant POLG was over expressed in MCF7 (transformed, non-invasive breast epithelial) cells under the control of a tetracycline responsive promoter. Mutations in POLG led to depletion of mtDNA which then resulted in decreased mitochondrial respiratory activity, decreased inner mitochondrial membrane potential, increased levels of reactive oxygen species (ROS). These alterations in mitochondrial function led to increased in vitro invasion, suggesting that shifting intracellular metabolism away from mitochondrial respiration by mtDNA depletion leads to enhanced tumorigenesis. Interestingly, the invasiveness of the cell was reverted when the mutant POLG gene was turned off and mtDNA returned to normal. Genetic alterations within the cell, including nicroRNA and mRNA expression were found when cells were expressing a mutant POLG. Furthermore, treatment with a methylation inhibitor restored POLG expression and mitochondrial function in breast cancer cells. Taken together, this report shows that POLG mutations are causing important metabolic & genetic alterations that may play a role in breast cancer.

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*breast cancer, mitochondrial DNA, tumorigenesis, polymerase gamma.

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Table of Contents

Final Report:

Introduction

Mutations in the mitochondrial DNA (mtDNA) polymerase, polymerase γ (POLG), are associated with a number of mitochondrial diseases[1], however, its role in currently cancer is unknown. There are three functional domain of POLG; the polymerase domain, linker domain, and exonuclease domain. Mutations that disrupt the polymerase or linker domain have been shown to result in the depletion of mtDNA content[2-6], whereas, mutations that disrupt the exonuclease activity of POLG result in an accumulation of mtDNA mutations[3,4,7]. Several human cancers have been found to have a decrease in mtDNA and an increase in mtDNA mutations[8], however, it is not clear whether this is casually related or if it is a result of genomic instability associated with cancer.

Body

POLG mutation in the polymerase domain alters mitochondrial function

A tetracycline responsive vector containing POLG with a mutation in the polymerase domain (POLG D1135A) was constructed as described in Singh et al 2009 (appendix) [9]. Our preliminary results showed that expression of POLG D1135A led to mtDNA depletion. By 5 days of expression the mtDNA was depleted by 80% (**Appendix fig 1B**). Depletion of mtDNA by POLG D1135A would be expected to affect the mitochondrial function. To determine this, ROS levels, mitochondrial membrane potential, and mitochondrial respiratory activity were measured as described in the attached manuscript. As **appendix fig 1C** shows, there was an increase in DHE oxidation after POLG D1135A expression. **Appendix fig 1D** shows that there is a 25% decrease in mitochondrial membrane potential in response to POLG D1135A expression. A growth curve was performed on these cells. POLG D1135A expressing cells grew slower than control cells with a doubling time of POLG D1135A cells was 68.6 h; whereas, the doubling time of control cells was 42.3 h. Mitochondrial respiratory activity was measured by the rate of resazurin reduction as previously described[10,11]. **Appendix fig 2A** shows that the re was a decrease in mitochondrial respiratory activity after POLG D1135A expression. This indicates that a there is a decrease in OXPHOS activity when mtDNA is depleted due to the POLG D1135A mutation. Since the mtDNA encodes for 13 subunits of OXPHOS, loss of mtDNA would be expected to decreas e oxidative metabolism. A decrease in mitochondrial OXPHOS activity was accompanied by a large

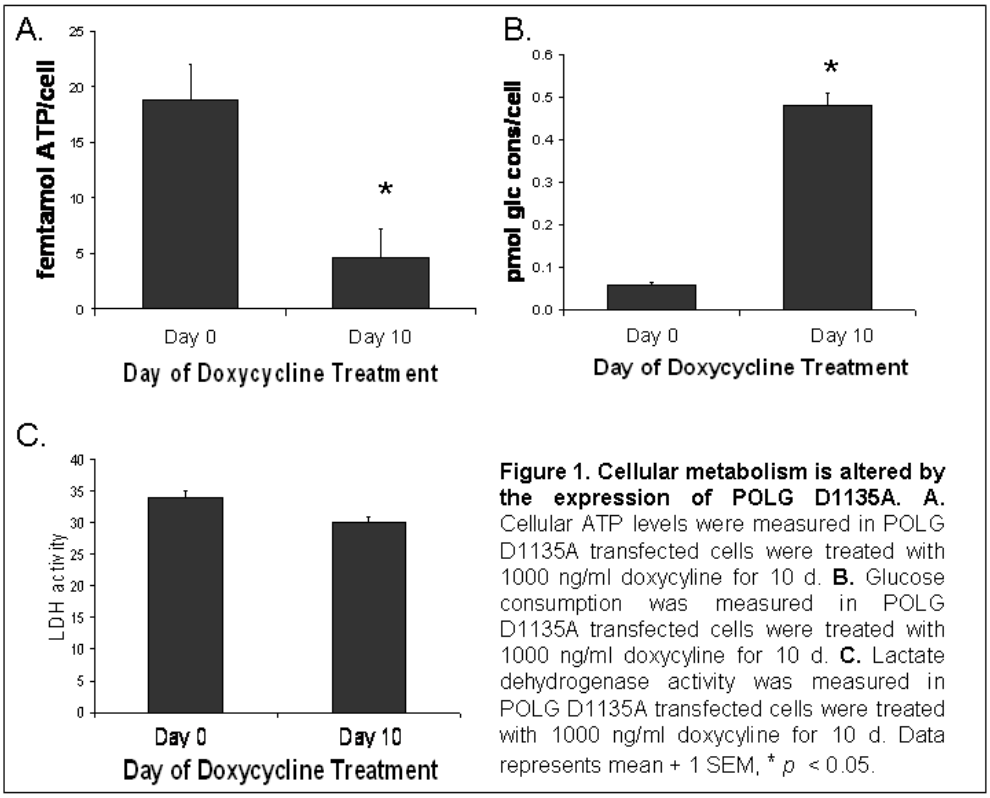

Figure 1. Cellular metabolism is altered by the expression of POLG D1135A. A. Cellular ATP levels were measured in POLG D1135A transfected cells were treated with 1000 ng/ml doxycyline for 10 d. **B.** Glucose consumption was measured in POLG D1135A transfected cells were treated with 1000 ng/ml doxycyline for 10 d. **C.** Lactate dehydrogenase activity was measured in POLG D1135A transfected cells were treated with 1000 ng/ml doxycyline for 10 d. Data represents mean + 1 SEM, * $p < 0.05$.

decrease in intracellular ATP levels. ATP was measured with the Sigma Somatic Cell ATP kit after 10 d of doxycycline treatment. MCF7 Tet-on cells expressing POLG D1135A had 25% the levels of ATP as compared to non-expressing cells (**Fig 1A**). We next measured glucose consumption from the cell culture media. According to the Warburg effect, cancer is accompanied by an increase in aerobic glycolysis[12]. Glucose in media samples was measured with a OneTouch Ultra LifeScan gluocometer. Glucose consumption increased 5 fold in POLG D1135A cells (**Fig 1B**). Lactate dehydrogenase (LDH) activity was measured by the reduction of NAD^+ in the presence of lactate and hydrazine. LDH activity was decreased slightly by POLG D1135A expression (**Fig 1C**).

POLG E1143G stable cells were tested for cellular metabolism and growth alterations as described above. ATP levels were decreased by 40% in POLG E1143G expressing cells (**Fig 2A**). Concurrently, there was a 1.6 fold increase in glucose consumption in these cells (**Fig 2B**). This indicates that there is a shift away from mitochondrial metabolism towards aerobic glycolysis. The activity of lactate dehydrogenase was deceased in a time dependent manner (**Fig 2C**). After 5 d of doxycycline treatment there was a significant decrease in LDH activity. We are still investigating why there is a large increase in glucose uptake, however, LDH activity is lower. The growth rate of POLG E1143G stable cells was as described above. The doubling time of doxycycline treated and untreated cells was 52.1 h and 32.5 h, respectively. By studying the metabolic processes and growth rates of these stable cells, we determined that expression of both polymerase domain mutants (D1135A and E1143G) result in a similar phenotype.

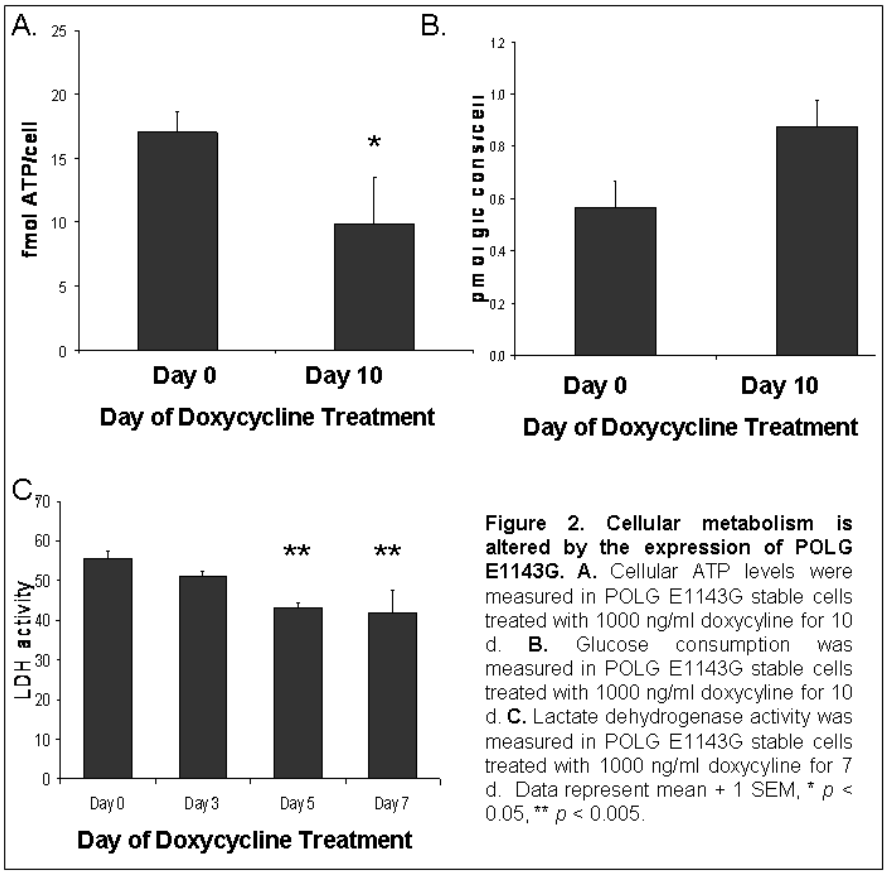

Figure 2. Cellular metabolism is altered by the expression of POLG E1143G. A. Cellular ATP levels were measured in POLG E1143G stable cells treated with 1000 ng/ml doxycyline for 10 d. **B.** Glucose consumption was measured in POLG E1143G stable cells treated with 1000 ng/ml doxycyline for 10 d. **C.** Lactate dehydrogenase activity was measured in POLG E1143G stable cells treated with 1000 ng/ml doxycyline for 7 d. Data represent mean + 1 SEM, * $p < 0.05$, ** $p < 0.005$.

We next wanted to determine what effect the expression of POLG mutations had on the *in vitro* invasiveness of MCF7 Tet-on cells. These cells, although transformed, are non-invasive. Our preliminary studies showed that by removing doxycycline and turning off the expression of POLG D1135A, mtDNA content is restored to normal after 7 d. In the next set of experiments we wanted to determine three things: 1) If overexpression of wild-type POLG leads to increased invasiveness, 2) If restoration of healthy mtDNA reversed the increase in invasiveness, 3) If mutations in other domains of POLG had the same effect. MCF7 Tet-on cells were transfected with POLG and treated with doxycycline. The cells were sorted for GFP on day 2 and grown in doxycycline for an additional 3 d (5 d with doxycycline). At day 5 the cells were plated in a Boyden chamber with 10% FBS as a chemoattractant. The remaining cells were placed in doxycycline-free media for an additional 7 d. On day 12 the cells

were harvested an assayed for Matrigel invasion as on day 5. Cells expressing a wild-type POLG had a non-significant decrease in Matrigel invasion, signifying that the overe xpression of the polym erase is not contributing to any in crease in invasiveness (**Fig 3A**). There is increase in *in vitro* invasiveness in MCF Tet-on cells expressing any of the POLG mutations after 5 d. When doxycycline was removed for 7 d (Day 12) all of the POLG m utants reverted back to norm al levels (**Fig 3 B-G**). This indicates that disruption of m tDNA helps govern th e invasive potential of these cells. The revers ibility of the invasiveness may be due to ep igenetic changes; hence we will look at epigene tic regulation such as microRNA expression and methylation status.

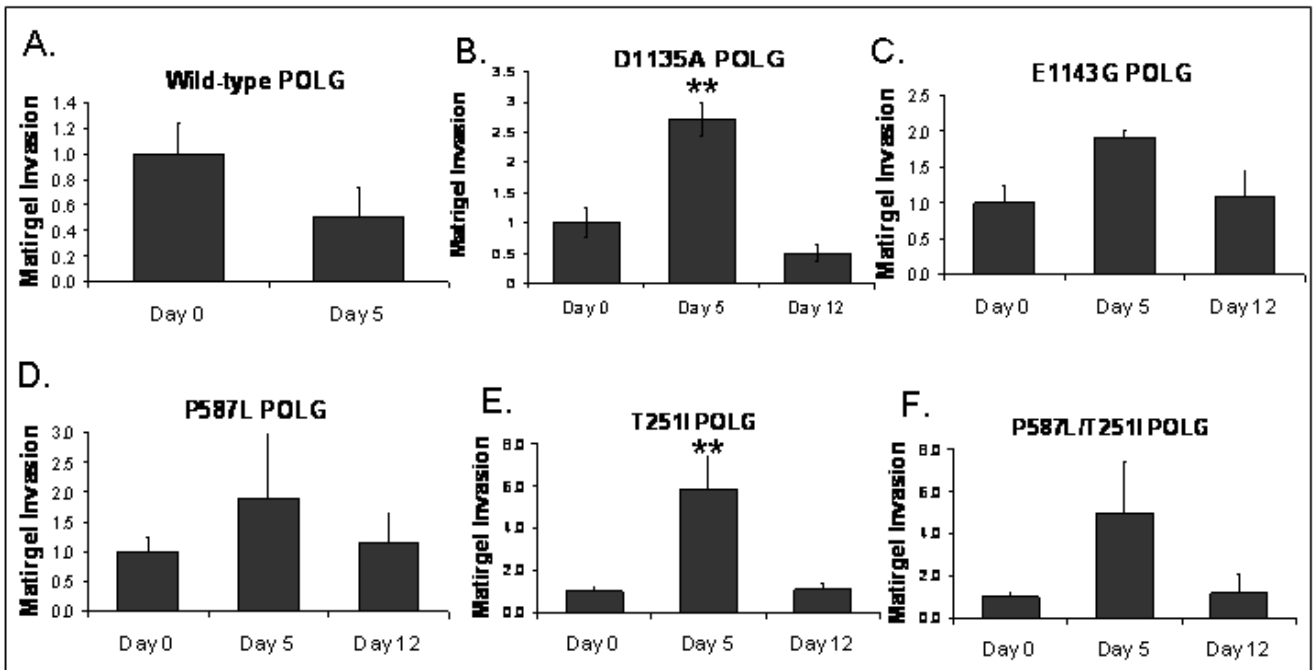

Figure 3. Mutations in POLG lead to increased *in vitro* invasion. Matrigel invasion of MCF7 Tet-on cells expressing a mutant POLG. Cell were treated with 1000 ng/ml doxycycline and sorted for GFP fluorescence. Cells were grown in the presence of doxycycline for 5 d, then the media was changed to doxycyline-free media for 7 additional d. Data represents mean percentage of invading cells normalized to day 0 ± 1 SEM, ** $p < 0.005$.

POLG D1135A causes an alteration in microRNA and mRNA expression

Our lab previously found that depletion of mitochondrial DNA by a chem ical method led to alterations in m icroRNA (miRNA) expression. To determine if genetically altering m tDNA by expressing a m utant POLG would al so result in differences in miRNA expression, we perform ed an Illumina human miRNA expression array on MCF7 Tet-on cells expressing POLG D1135A. Cells were transfected and sorted for GFP as described a bove. MiRNA expression at day 0 was compared to expression at day 5. 57 m iRNAs were upregulated in POLG D1135A expressing cells with a log2 ratio greater than 2, and 47 m iRNAs were down regulated with a log2 ratio less than -2. After 5 d of doxycycline treatment, doxycycline free m edia was added to subset of the cells for an add itional 7 d. Previously, we have shown that this is am ple time to allow mtDNA content to be fully restored and the invasive potential to return to normal. The altered miRNA expression seen at day 5 was also returned to normal. In fact, only one miRNA had a log2 ratio less than -2. To determine if mitochondrial mutations as a result of a POLG e xonuclease domain mutation may also lead to a difference in miRNA expression we transiently expressed POLG T2 51I and sorted for GFP as before . Using the sam e experimental parameters as described above, we saw no significant up or down-re gulation of miRNA between day 0 and day 5. When comparing day 0 and day 12, there were 3 miRNA that were up-regulated.

Because the expression of POLG D1135A in MCF7 Tet-on cells altered the miRNA profile, we were interested in determining the change in gene expression in these cells. We performed an Illumina v.H12 microarray on POLG D1135A cells at 0 and 5 d of doxycycline treatment. 13 genes were up-regulated in doxycycline treated cells and 15 genes were down-regulated. As expected there was a 9 fold increase in POLG expression (log2 ratio = 3.1). Therefore, we are able to show that depletion of mitochondrial DNA by expression of POLG D1135A leads to global expression changes in breast cells.

POLG in breast cancer cells

To determine if POLG expression is altered in breast cancer cell lines, we screened a panel of breast cancer cells lines for POLG expression. As **fig 4A** shows, MDAMB231 cells have a diminished expression of POLG. These cells are the most aggressive of the breast cancer cells. Also, the rho^0 cells (devoid of mtDNA) decreased expression of POLG. We then looked at the mtDNA content of few of the cell lines and found that the MCF7 cells had a very slight decreased in mtDNA content, whereas, the MDAMB231 cells had a significant decreased in mtDNA content (**Fig 4B**). The mtDNA content matched the expression of POLG these breast cancer cells.

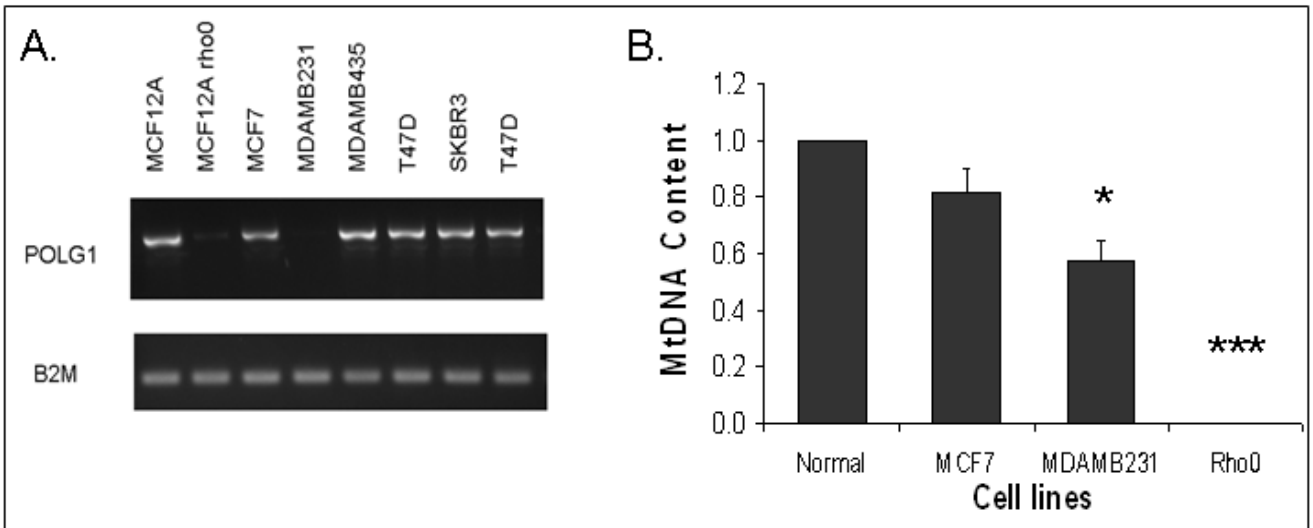

Figure 4. POLG expression and mitochondrial DNA content in breast cancer cells. A. RT-PCR of POLG expression in breast cancer cells. B2M serves as a loading control. **B.** Mitochondrial DNA content in breast cancer cells was measure by real-time PCR. The ratio of mtDNA to nuclear DNA was used as an index for measuring the mtDNA content. Data represent mean + 1 SEM, * $p < 0.05$, *** $p < 0.0005$.

Transgenic mouse model

In our proposal we proposed to make a transgenic mouse model that will express POLG mutations in breast tissue when induced with doxycycline. To do this we prepared a DNA fragment with that contains the tetracycline responsive element and the POLG gene with a polymerase domain mutation (D1135A). This gene fragment was injected into C57BL/6 embryos by the Roswell Park Cancer Institute Gene Transfer and Transgenics Core Facility and the embyos were transplanted into a mother mouse. The resulting litters were genotyped by tail DNA. 2 positive transgenic mice were identified, one male mouse (7610) and one female mouse (7619) (**Fig 5**). The founder mice (G_0 generation) were breed to C57BL/6J mice from Jackson Laboratory to establish the F_1 generation. The transgenics female mouse gave birth to 8 pups and the male transgenics mouse fathered 5 pups. When

the pups are at weaning age they will be genotyped by tail DNA, and positive siblings will be breed to each other to establish the F_2 generation.

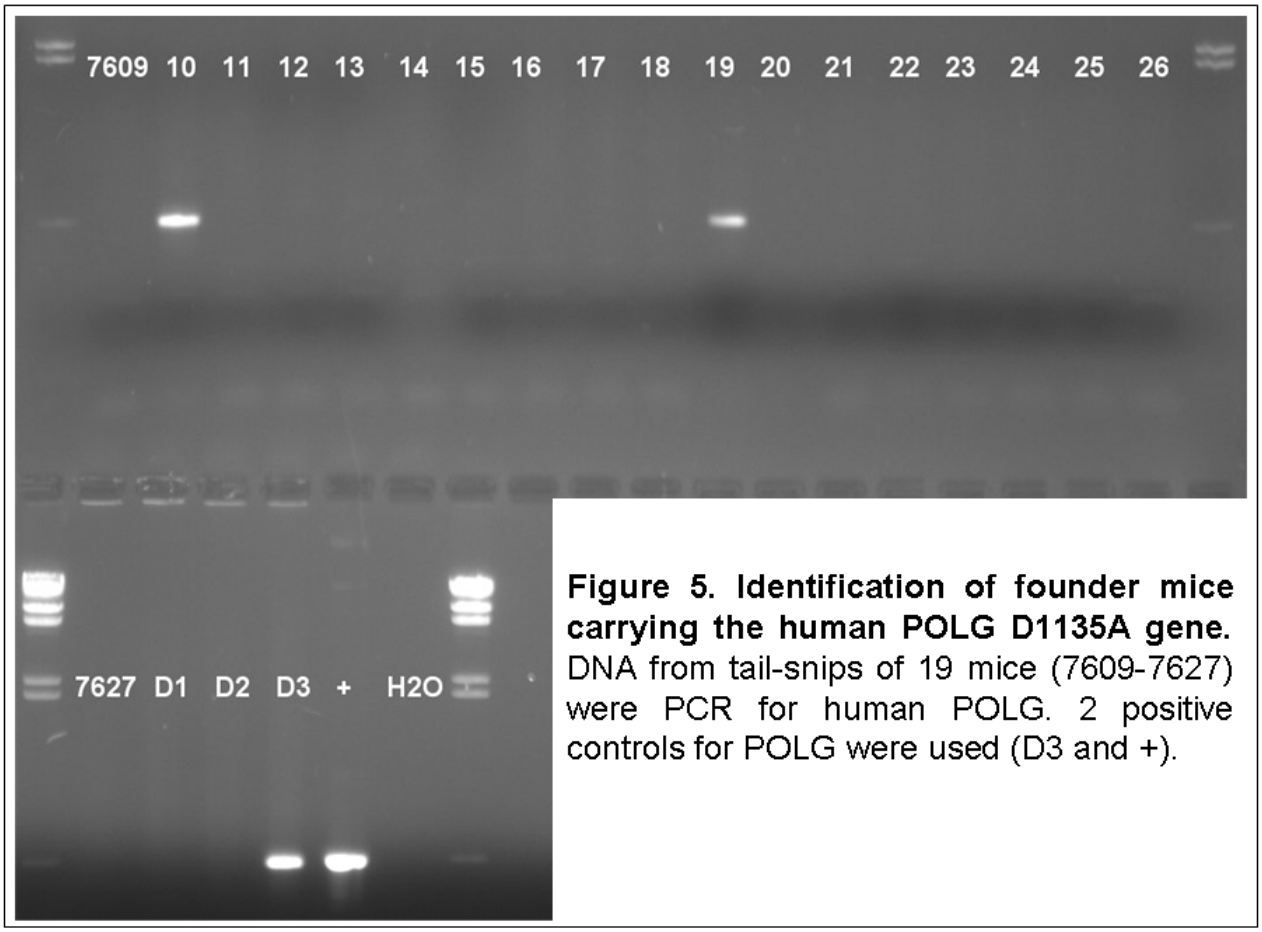

Figure 5. Identification of founder mice carrying the human POLG D1135A gene. DNA from tail-snips of 19 mice (7609-7627) were PCR for human POLG. 2 positive controls for POLG were used (D3 and +).

Key Research Accomplishments

- Showed mitochondrial dysfunction with POLG D1135A and POLG E1143G expression
- Showed increased *in vitro* invasion by expression of POLG mutants
- Showed reversibility of invasiveness by stopping the expression of POLG mutants
- Showed altered microRNA expression profile for POLG D1135A that is reversed when expression of POLG D1135A is stopped
- Identified gene expression differences in POLG D1135A expressing cells

Reportable Outcomes

This work along with previous studies from our laboratory has led the publication of a manuscript entitled "Mutations in mitochondrial DNA polymerase γ promote breast tumorigenesis" in the *Journal of Human Genetics*[9]. Additionally, an abstract entitled "Contribution of polymerase γ mutations to the Warburg effect and its role in cancer" was submitted to the United Mitochondrial Disease Foundation (UMDF) for their Mitochondrial Medicine 2010 International Symposium. This abstract was selected for an abstract presentation at the meeting in Scottsdale, Arizona as was presented by Dr. Owens. This work has also led to the establishment of stable MCF7 Tet-on POLG E1143G cells

that will express POLG with an E1143G mutation when treated with doxycycline, and the establishment of C57BL/6 TRE-mPol founder mice that carry a doxycyline responsive mutant human POLG gene.

Conclusions

Our laboratory has recently shown that mutations in POLG are associated with cancer[13]. This is first study to examine the effect of POLG on the cellular processes of tumorigenesis. The work conducted in the past year has shown that mutations in the polymerase domain of POLG change the metabolism of the cell, resulting in increased invasiveness and miRNA and mRNA expression changes. This work helps define how mitochondrial changes can lead to more global cellular changes that lead to tumorigenesis.

Our data show that the malignant phenotype of nonaggressive breast cancer cells (MCF7) that was induced by a POLG mutation is reversible by removing the mutant gene and allowing the mtDNA to be replenished (Fig 3). Therefore, through the use of gene-specific drugs or genetic engineering promotion and progression of breast cancer in women with a POLG mutation may be reversed. This not only would apply to women with a POLG mutation, but may also be promising to patients whose mtDNA has been depleted by other mechanisms.

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Mutations in mitochondrial DNA polymerase γ promote breast tumorigenesis

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Abstract

Decreased mitochondrial oxidative phosphorylation (OXPHOS) is one of the hallmarks of cancer. To date the identity of nuclear gene(s) responsible for decreased OXPHOS in tumors remains unknown. It is also unclear whether mutations in nuclear gene(s) responsible for decreased OXPHOS affect tumorigenesis. Polymerase γ (POLG) is the only DNA polymerase known to function in human mitochondria. Mutations in POLG are known to cause mtDNA depletion and decreased OXPHOS resulting in mtDNA depletion syndrome (MDS) in humans. We therefore sequenced all coding exons [2-23] and flanking intron/splice junctions of POLG in breast tumors. We found that the POLG gene was mutated in 63% of the breast tumors. We identified a total of 17 mutations across the POLG gene. Mutations were found in all three domains of POLG protein, including T251I (exonuclease domain), P587L (linker region) and E1143G (polymerase domain). We identified two novel mutations that include one silent (A703A) and one missense (R628Q) mutation in the evolutionarily conserved POLG linker region. Additionally, we identified three novel mutations in the intronic region. Our study also revealed that mtDNA was depleted in breast tumors. Consistently, mutant POLG when expressed in breast cancer cells induced depletion of mtDNA, decreased mitochondrial activity, decreased mitochondrial membrane potential, increased levels of reactive oxygen species (ROS), and increased matrigel invasion. Together, our study provides the first comprehensive analysis of the POLG gene mutation in human cancer and suggests a role for POLG in 1) decreased OXPHOS in cancers and 2) in promoting tumorigenicity.

Keywords

Breast Cancer; POLG; MtDNA; Mitochondria; Mutation; Mitochondrial

INTRODUCTION

Decreased mitochondrial oxidative phosphorylation (OXPHOS) is one of the most common and profound phenotypes of cancer cells [1-10]. As early as 1930, the German biochemist Otto Warburg described OXPHOS differences in the mitochondria of tumor versus normal cells [1, 2]. He proposed that cancer initiates from irreversible injury to OXPHOS[3]. He further proposed that decreased OXPHOS led to an increase rate of aerobic glycolysis in most cancers. This phenomenon is described as the Warburg Effect.

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In human cells the OXPHOS system is assembled from 13 mtDNA (mitochondrial DNA) genes and over 85 nDNA (nuclear DNA) genes. The entire mitochondrial genome is devoted to the production of 13 protein subunits of OXPHOS complexes (I, III, IV and V) involved in respiration and ATP synthesis. We investigated the underlying reason for decreased OXPHOS in breast cancer and discovered that more than 40% of primary breast tumors lack detectable expression of cytochrome c-oxidase subunit II (OXPHOS complex IV) encoded by mtDNA [11]. Other laboratories have measured mtDNA content directly in tumors and report a decrease in mtDNA content in breast, renal, hepatocellular, gastric and prostate tumors[12-17]. Depletion of mtDNA is also supported by a decrease in OXPHOS levels in renal tumors [18]. It is also noteworthy that drugs used for treating HIV inhibit POLG, which in turn induces mtDNA depletion [19]. Tamoxifen, a commonly used drug for the treatment of breast cancer, also depletes mtDNA [20]. A recent study also demonstrates that the depletion of mtDNA correlates with tumor progression and prognosis in breast cancer patients [21]. To date the identity of nuclear gene(s) responsible for mtDNA depletion and decreased OXPHOS in tumors remains unknown. It is also unclear whether mutations in nuclear gene(s) involved in mtDNA depletion and decreased OXPHOS affect tumorigenesis.

The first mtDNA depletion syndrome (MDS) was described more than 15 years ago [22]. MDS results from mutation(s) in nuclear-encoded genes that participate in mtDNA replication, in mitochondrial nucleotide metabolism and in the nucleotide salvage pathway. So far, only six MDS genes have been identified. These nuclear genes include: mtDNA polymerase gamma (POLG), mtDNA helicase twinkle [23], thymidine kinase 2 (TK2) [24,25] deoxyguanosine kinase (DGUOK) [26,27], SUCLA2, the gene-encoding beta-subunit of the adenosine diphosphate-forming succinyl coenzyme A synthetase ligase [28,29], and MPV17, a mitochondrial inner membrane protein [28,30]. Of these nuclear genes, POLG is the most frequent target of mutation, and is involved in a variety of mitochondrial diseases. To date, more than 150 mutations in POLG have been identified [31].

POLG is the only DNA polymerase known to date in human mitochondria. POLG is essential for the development of an embryo [32]. It contains a large catalytic subunit, POLG (140-kDa), and two smaller identical accessory subunits, POLG2 (55-kDa)[33]. POLG belongs to the family of A type DNA polymerases [34] consisting of an exonuclease domain with three exo motifs, I, II and III, and a polymerase domain with three pol motifs, A, B and C, along with an intervening linker region [35]. As with any other polymerase, POLG has been involved in DNA polymerase, 3' to 5' exonuclease and the 5'dRP lyase activities of mtDNA replication [36].

The POLG gene maps to 15q25, is 21kb in size and consists of 23 exons. POLG contains CAG trinucleotide repeats that code for polyglutamine in the second exon, which is not present in any of the polymerases or orthologs [37]. Since the first identification of POLG mutations in PEO, it has become evident that mutations in POLG are a major cause of many human diseases, ranging from Alpers syndrome to male infertility, Parkinsonism and other mitochondrial diseases [36,38-41]. Most disease phenotypes associated with mutations in the POLG are due to mutations and/or depletions in mtDNA.

In this study, we analyzed POLG gene mutations and the associated reduction in mtDNA content in breast tumors. We performed mutational analyses of all coding exons and flanking intron/splice junctions of POLG. This study reports novel somatic mutations in POLG that are frequently found in breast cancer. In addition we provide evidence that mutations in POLG gene promote tumorigenesis.

MATERIALS AND METHODS

Tumor Samples

Tissue samples were collected from the patients with breast tumors undergoing surgery for treatment at the Roswell Park cancer institute and from Cooperative Human Tissue Network (CHTN) with the informed consent.

Cell culture

The breast cell lines MCF7, MDAMB231 and control cell lines MCF12A, MCF12ARho0 were grown in Dulbecco's modified Eagle's media (Cellgro, Herndon, VA, USA) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). MCF7 Tet-On Advanced cells (Clontech, Mountain View, CA, USA) were grown in DMEM supplemented with 10% Tet System Approved FBS (Clontech), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen), 100 μg/ml G418 (Cellgro) and 50 μg/ml uridine (Sigma, St. Louis, MO, USA). Cells were maintained in a 37°C, 5% CO_2 environment.

Plasmid construction and site directed mutagenesis

The full length POLG cDNA was subcloned into the inducible mammalian expression vector pTRE-Tight-BI-AcGFP1 (Clontech). Site directed mutants were created for the mutations T251I (Exonuclease domain); P587L (Linker domain); T251I and P587L (Double mutant); D1135A and E1143G (Polymerase domain) using the site directed mutagenesis kit (Stratagene/ Agilent, Santa Clara, CA, USA). Mutations were confirmed by sequencing the complete ORF of each mutant clones. The primer sequences used for site directed mutagenesis are as follows with the mutated site in upper case:

T251I_F: 5'ccctggaggtccctaTtggtgccagcag 3'

T251I_R: 5'ctgctggcaccaAtagggacctccaggg 3'

P587L_F: 5' tgcatggacccTgggccccagcc 3'

P587L_R: 5' ggctggggcccAgggtccatgca 3'

D1135A_F: 5' gcatcagcatccatgCGgaggttcgctacctgg 3'

D1135A_R: 5' ccaggtagcgaacctcCGcatggatgctgatgc 3'

E1143G_F: 5' cctggtgcgggGggaggaccgct 3'

E1143G_R: 5'agcggtcctccCcccgcaccagg 3'

POLG gene mutational analyses

DNA was isolated from tumors and cell lines with the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). All 23 exons and flanking intron/splice junctions of POLG were amplified by PCR with AmpliTaq Gold-polymerase. The primers and PCR conditions are given in the Supplementary Table 1. The PCR products were checked by agarose gel electrophoresis, purified by the QIAEX II Gel Extraction Kit (Qiagen) and sequenced using the BigDye terminator Ready Reaction Kit v.3 on a 3100 Genetic Analyzer Automatic Sequencer (Applied Biosystems, Foster City, CA, USA).

Mitochondrial whole genome sequencing

Complete mtDNA of four representative samples was amplified using the 24 sets of overlapping primers. Direct Sequencing of PCR products were carried out using 100.0 ng of PCR product. The mitochondrial DNA mutations were identified by comparing the sequences with rCRS.

Analysis of mtDNA content

MtDNA content was measured in breast tumor samples and cell lines by SYBR green method (SA biosciences, Frederick, MD, USA) in 7900HT Fast Real time PCR system (Applied Biosystems). Standard curves were obtained using the MCF12A cell line DNA and the reactions were performed in triplicates. Two sets of primers, one amplifying mtDNA tRNA (Leu) gene and other amplifying the nuclear DNA (Beta 2 microglobulin) were used. The ratio of the mtDNA compared to the nuclear DNA was used an index for measuring the mtDNA content [42].

MCF7 Tet-On Advanced cells were transiently transfected with pTRE-Tight-BI-AcGFP1 POLG D1135A vector according to the Fugene HD Transfection Reagent protocol (Roche, Basel, Switzerland). Media containing 1000 ng/ml doxycycline (Clontech) was added 4 h post-transfection. Transiently transfected cells were harvested 2 d after doxycycline treatment and sorted for GFP positive cells on a BD FACAria cell sorter (Becton Dickinson Biosciences, Franklin Lakes, NJ). GFP positive cells were replated with 1000 ng/ml doxycycline. DNA was isolated with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. mtDNA content was analyzed as described above.

GFP Induction

The mean fluorescent intensity of GFP was determined reading the fluorescence of pTRE-Tight-BI-AcGFP1 transfected cells on the FL1 channel of a FACSCalibur (Becton Dickinson Biosciences). Values are represented as mean fluorescence intensity.

Mitochondrial Functional Analyses

MCF7 Tet-On Advanced cells were transiently transfected with pTRE-Tight-BI-AcGFP1 POLG D1135A vector according to the Fugene HD Transfection Reagent protocol. Media containing 1000 ng/ml doxycycline was added 4 h post-transfection. Expression of POLG D1135A was induced by 1000 ng/ml doxycycline for up to 5 days. Cells were analyzed for reactive oxygen species (ROS) production by labeling with 10 μM dihydroethidium (DHE) for 40 min. Mitochondrial membrane potential was assessed by labeling the cells with 100 nM tetramethylrhodamine, ethyl ester, perchlorate (TMRE) for 35 min. Fluorescence of both dyes were analyzed on a FACSCalibur and gated for GFP positive cells.

Mitochondrial respiratory activity was measured by the rate of resazurin reduction as previously described [43,44]. MCF7 Tet-On Advanced cells were transiently transfected with the pTRE-Tight-BI-AcGFP1 POLG D1135A vector, treated with 1000 ng/ml doxycycline, and sorted for GFP positive cells as described above. Cells assayed for mitochondrial respiratory activity as measured by the change in resazurin reduction.

Matrigel Invasion Assay

MCF7 Tet-on cells were transfected with mutant POLG plasmid were treated with 1000 ng/ml doxycycline and sorted for GFP as described above. 5 d post-doxycycline treatment cells were analyzed for *in vitro* matrigel invasion. Cells were plated in serum-free media in an upper Boyden chamber with a Matrigel membrane. Complete media containing 10% FBS was added

to the bottom well as a chemoattractant. Cells in the chamber were incubated for 24 h and the membrane was fixed and stained with the Diff-Quick Stain Set.

RESULTS

Mutation in POLG polymerase increase tumorigencity of breast cancer cells

To determine the functional as well as tumorigenic role of POLG, a mutant defective in polymerase domain (D1135A) was cloned under tetracycline inducible promoter and expressed in MCF7 breast cancer cell line. A bicistronic promoter provided the expression of both GFP and POLG simultaneously. GFP expression was used as a guide to identify cells expressing the mutant POLG gene (Figure 1A). The mtDNA content was drastically reduced when expression of D1135A POLG mutant was turned on with addition of doxycycline (Figure 1B).

These studies demonstrate that mutation(s) in the POLG polymerase domain lead to reduced mtDNA content. We therefore characterized the effect of POLG mutations on mitochondrial function. As figure 1C shows, there is a 2-fold increase in the level of ROS as measured by DHE oxidation 2 d after POLG1 D1135A expression. This change in DHE oxidation decreases by day 5, potentially indicating a shift away from oxidative phosphorylation as a metabolic source. The majority of ROS production in the cell comes from Complex I and Complex III of oxidative phosphorylation. Figure 1D shows that there is a 25% decrease in mitochondrial membrane potential in response to POLG D1135A expression. Mitochondrial respiratory activity was measured by the rate of resazurin reduction as previously described [43, 44]. Resazurin is a redox-active dye that acts as an electron acceptor at Complex IV of the ETC and fluoresces upon reduction[44]. Expression of POLG D1135A causes a decrease in oxidative phosphorylation when mtDNA is depleted from the POLG D1135A mutation (Figure 1E). Since the mtDNA encodes for 13 subunits of oxidative phosphorylation, loss of mtDNA would be expected to decrease oxidative metabolism. We then measured the *in vitro* tumorigeneic phenotype of cells expressing mutant POLG by Matrigel Invasion Assay. Figure 1F shows that cells expressing D1135A mutant POLG were more invasive than the vector alone control. We conclude that mutations in the polymerase domain of the POLG gene causes depletion of mtDNA, decreases mitochondrial membrane potential, decreases mitochondrial activity and increases oxidative stress which together promotes tumorigenesis.

POLG mutations identified in primary breast tumors

We screened all the coding exons and intron/splice junctions of POLG in 19 breast tumor samples and three cancer cell lines (Supplementary Table 1). The sequence variants found are summarized in Table 1 and depicted in Figures 2A and 2B. We identified novel as well as previously described pathogenic mutations in POLG [33, 45]. The electropherograms of key mutations are given in Supplementary Figure 1. In exon 2 of POLG, CAG repeats, was found to be extended in four breast tumor samples. We detected c.752C>T in exon 3 that affects the exonuclease domain of the protein (T251I), which was reported in PEO and infantile hepatocerebral syndrome [33]. Four mutations were detected in exons 9 and 10, which encode the linker region, including two novel and two previously reported mutations. The novel variants include c.1883G>A, a missense mutation causing change in the conserved amino acid Arginine to Glutamine at the 628 residue of POLG protein (Figure 2C), and another that is a silent mutation. Mutations were found in exon 16 (c.2492A>G) and exon 21 (c.3428A>G), which encode the POLG polymerase domain. In addition, we identified three novel variants in the intron/splice junctions of POLG. These results suggest that the POLG gene is a frequent target of mutation in breast tumors.

MtDNA mutations in primary breast tumors

Mutations in the POLG gene are known to result in the accumulation of mutations in mtDNA [46]; therefore, we sequenced the entire mitochondrial genome of four representative tumors samples. Interestingly, in all four samples analyzed, the mutations were concentrated in the control D-loop region (Table 2). These mutations have previously been shown to occur in a variety of tumors [47-51]. These results suggest that the identified POLG mutation in breast tumors frequently targets the D-loop region.

Reduced mtDNA content in primary breast tumors and cell lines

In addition to mutations in mtDNA, a common consequence of POLG mutation in mitochondrial diseases is mtDNA depletion[33]. MtDNA depletion is also found in breast tumors and is associated with the prognosis of breast cancer [21]. To identify the effect of the POLG mutations described above in breast tumors, we measured the mtDNA content by real-time PCR. The single copy nuclear gene β2microglobulin was used to normalize the mtDNA content. Rho0 cells devoid of mtDNA served as a negative control. Figure 3A shows the mtDNA content index in primary breast tumors. MtDNA content was reduced in samples containing the POLG mutation. Interestingly, a similar observation was made in breast cancer cell lines (Figure 3B). We conclude that POLG mutation leads to decrease in mtDNA content.

Breast Tumor POLG mutations promote tumorigenesis

The above study demonstrates that the POLG gene is frequently mutated in primary breast tumors (Figure 1). Therefore, by using site-directed mutagenesis we mutagenized the cDNA encoding representative mutations identified in breast tumors in all three functional domains of POLG (E1143G - polymerase domain, P587L - linker domain, and T251I - exonuclease domain), as well as the double mutations P587L and T251I that is often found in *cis*. Each mutant was tested for *in vitro* invasion 5 d after doxycycline treatment. Using the Matrigel invasion tumorigenicity assay, we demonstrate that expression of mutant POLG leads to increased invasiveness *in vitro* (Figure 4). These results suggest that POLG mutations identified in breast tumor indeed promote tumorigenesis by increasing the invasive potential of breast cancer cells.

DISCUSSION

Although mutation(s) in the POLG gene are shown to result in decreased OXPHOS, decreased mtDNA content and the pathogenesis of human mitochondrial diseases, its role in the pathogenesis of cancer is unclear. Therefore, we screened all coding exons and intron/splice junctions of POLG for mutations in breast tumors. Our analysis identified novel mutations in POLG. We also identified previously described mutations that are known to be involved in the pathogenesis of many mitochondrial diseases. Mutations were found in all three domains of the POLG protein. We identified a mutation in the exonuclease domain (C752T) of the breast tumor that is associated with PEO and infantile hepatocerebral syndrome [52-54].

Several mutations in the POLG linker region that lead to neuromuscular diseases, including Alpers's disease and Parkinson's disease have been described [35,55]. However, we identified two novel linker region mutations in breast tumors. These include: (a) a missense mutation in the evolutionarily conserved (R628Q) linker region and (b) a silent linker region mutation (A703A). Previous functional analysis of the linker region mutants shows decreased enzyme activity, DNA binding and processivity of the polymerase[56]. The mutants in the linker region of the fruit fly enzyme also affect its enzyme activity, processivity and DNA-binding affinity [57]. The codon usage analysis for human POLG suggest that 56/103 Alanines use the GCC codon, but only 13/103 alanines use the GCA codon. This is important in the context of identified c.2109C>A (A703A) substitution in the Linker region. It is conceivable that base

substitution causes ribosome stalling because Alanyl-tRNAs don't recognize the GCA codon so well which may slow the synthesis of protein. In some proteins, this type of substitution results in improper folding of protein leading to reductions in activity.

Breast tumors also harbored mutations in the polymerase domain (Y831C and E1143G) of POLG. Previous studies suggest that these mutations inhibit mtDNA polymerase activity and, hence, may lead to mtDNA depletion[58]. Targeting POLG polymerase mutations in mice hearts also provides *in vivo* evidence for the depletion of mtDNA [59].

One of the common features associated with mitochondrial diseases is the co-occurrence of mutations in POLG. The mutation T251I is found to occur in *cis* with P587L in many mitochondrial diseases [34]. Likewise, T251I was found in *cis* with P587L in two breast tumors. However, the E1143G mutation, frequently found in conjunction with W748S in ataxia[60], was uniquely present in breast tumors. POLG contains trinuleotide repeats (CAG) in the coding region [37]. CAG trinucleotide repeat sequences are highly unstable, leading to the expansion or contraction of the repeat sequence, and are known to be involved in the pathogenesis of many human diseases [61]. Our study revealed that the expansion of CAG repeats in more than 20% of breast tumors analyzed.

We also identified novel intron/splice junction variants in conjunction with CAG repeats. Mutations in the intron/splice junctions of other genes are known to induce exon skipping, activation of the cryptic splice sites or alteration of the balance of the alternative spliced isoforms [62]. Variants in the splice junctions, particularly the GTAG insertion into intron 17, are predicted to alter splicing and POLG activity, as is also observed in PEO patients [63,64]. The CAG in 43-55Q was found to co-occur with seven variants in the intron/splice junction in two breast cancer cases. Interestingly, all breast tumors with CAG repeat expansion contained at least one splice site variant c.2734+39 insGTAG. POLG repeat expansion is reported to be associated with testicular cancer [65]. The POLG CAG repeats variation is also a predisposing genetic factor in idiopathic sporadic Parkinson's disease [55]. The expansion of CAG located in number of genes has been shown to cause many dominantly inherited neurodegenerative diseases, described as polyglutamine diseases [66]. The CAG repeats variation in other genes, such as androgen and estrogen receptors, plays an important role in breast and other cancers [67-69]. The contraction of CAG repeats in POLG affects its expression [58]. However, it is unknown at this time whether the expansion of CAG repeats in the POLG gene described in this paper affects its expression. An expanded CAG tract seems to affect the function of the host protein through protein-protein interaction [66]. It is conceivable that CAG expansion in POLG affects its function and may contribute to tumorigenesis. However, further studies are required to identify the exact role of POLG CAG expansion in cancer.

Mutations in POLG are known to deplete mtDNA in multiple tissues of mitochondrial disease patients [70]. Interestingly, our analysis also revealed 1) decreased mtDNA content in primary breast tumors and 2) when mutant POLG was expressed in breast cancer cells it led to depletion of mtDNA. Furthermore we identified mutations that were predominantly present in the D-loop control region of mtDNA. An increased incidence of novel mtDNA point mutations has been demonstrated in patients with POLG mutations[71,72]. The highest incidence of the mtDNA D-loop mutations could be due to the mutations affecting exonuclease and the polymerase domains of POLG. These findings suggest that reduced mtDNA content in breast tumors may arise due to 1) inefficient enzyme activity associated with POLG mutations and/or 2) mutations in the D-loop region affecting the binding of nuclear factors involved in mtDNA replication. Irrespective of POLG-induced depletion, our studies[11,73] and those of others[74,75] suggest that mtDNA depletion leads to tumorigenicity. Indeed, we recently demonstrated that depletion of mtDNA in breast epithelial cells lead to neoplastic transformation, and that this process is mediated by p53 [9]. These studies led us to ask whether POLG mutations, particularly the one

in the polymerase domain that causes mtDNA depletion play a role in tumorigenesis. Studies presented in this paper demonstrate that D1135A polymerase domain mutant when expressed in MCF7 cells functions as dominant negative and promote tumorigenesis *in vitro.* We also show that expression of mutant protein results in decreased mtDNA content, decreased OXPHOS, decreased mitochondrial membrane potential and increased oxidative stress which together contribute to increased tumorigenic phenotype. We also asked whether other POLG mutations play a role in tumorigenesis. The data presented in this paper show that with the exception of linker domain mutation (P587L), all other mutants (Polymerase domain E1143G; and exonuclease domain T251I) show increased tumorigenicity in breast cancer cells. Since mutations P587L and T251I are often found in *cis* in many mitochondrial diseases we also determined the effect of double mutant on Matrigel invasion. Our results show lack of synergistic effects on tumorigenicity in double mutants. The single T251I mutant was as invasive as the double P587L/T251I mutant. These studies suggest that P587L is not a significant player towards increased invasive property of MCF7 cells.

Apart from depletion, breast tumors contained mutations in mtDNA. Mutations in POLG are known to cause mutations in mtDNA. The mtDNA mutator mice that harbor the mutation in the exonuclease domain (that abolishes the POLG proof reading activity) show a marked reduction in lifespan due to the increased rate of mtDNA mutation [46, 76]. To date, there is no published report that describes the incidence of tumor development in these mice. It is possible that mtDNA mutations observed in these mice do not initiate tumorigenesis, i.e., transform normal cells, but rather are involved in the promoting tumoringenesis (as described in this paper) once cells are transformed. This argument is substantiated by our report which demonstrates that mtDNA mutations in normal cells do not confer tumorigenicity. In contrast, mutant mtDNA from breast tumors when transferred to transformed cells show metastasis [77]. In summary, our studies described in this paper provide the first comprehensive analyses of POLG gene mutations in human cancer that suggest a role for POLG in human tumorigenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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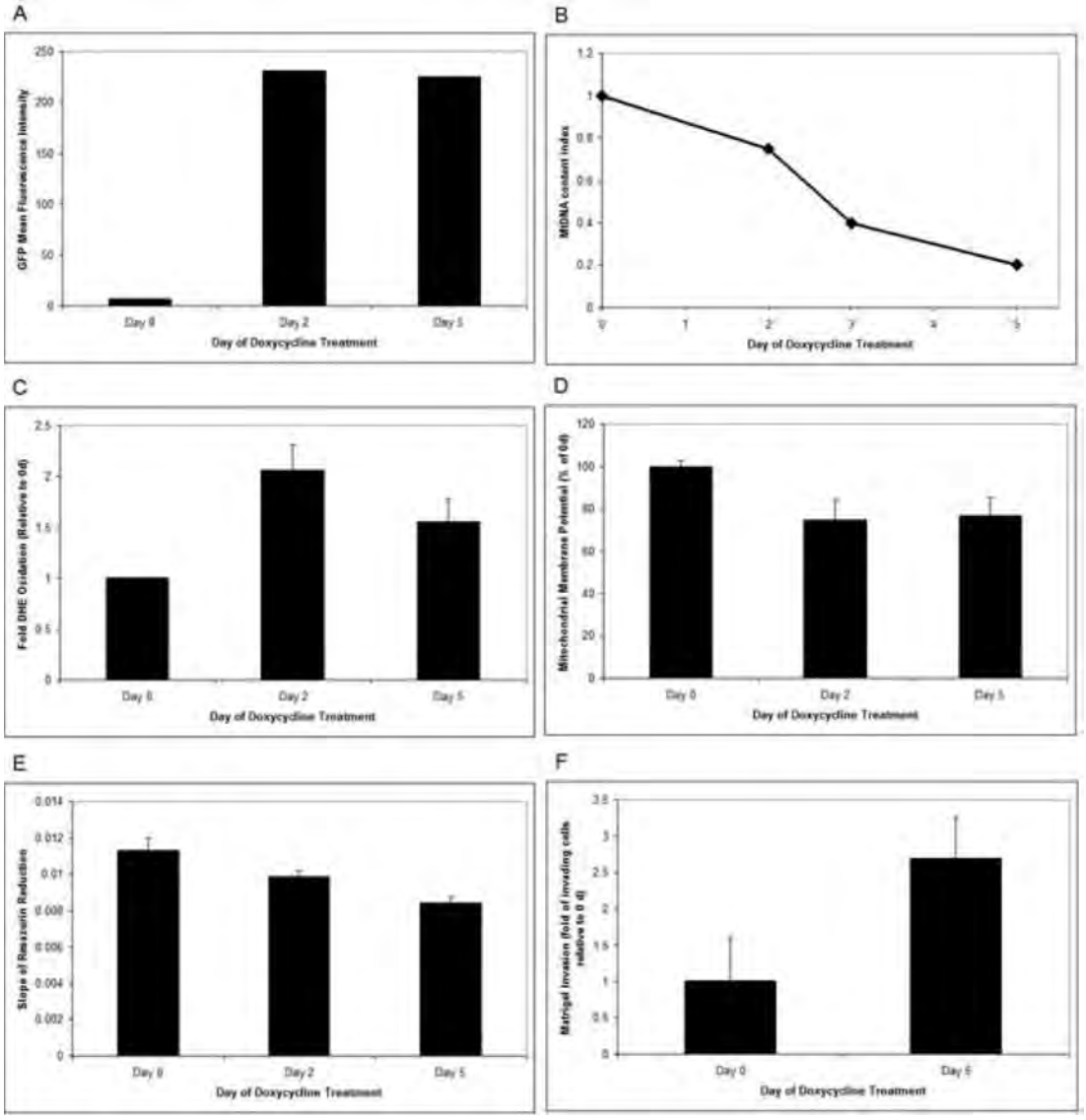

Figure 1. POLG D1135A mutant depletes mtDNA and promotes tumorigenicity in breast cancer cells

POLG D1135A cDNA was cloned in the tetracycline inducible plasmid pTRE-Tight-BI-AcGFP1. A bicistronic promoter provided the expression of both GFP and POLG simultaneously. Transfected MCF7 Tet-on Advanced cells were treated with 1000 ng/ml doxycycline for up to 5 day and were sorted by FACS. **A)** GFP fluorescence was used as a guide to sort cells expressing the mutant POLG gene. Mean fluorescent intensity was determined on the FL1 channel of a FACSCalibur flow cytometer. Data represent geometric mean fluorescence intensity. **B).** MtDNA index in MCF7 Tet-on Advanced cells expressing POLG D1135A. The ratio of mtDNA to nuclear DNA was used as an index for measuring the mtDNA content **C).** DHE oxidation of MCF7 Tet-on Advanced cells containing POLG D1135A was measured. Mean fluorescence intensity of each treatment group was normalized to day 0 and expressed as fold DHE oxidation + 1 SD.**D)**. Mitochondrial membrane potential was measured by TMRE fluorescence. Data represents mitochondrial membrane potential as a percent of control (day 0) + 1 SD. **E)**. Mitochondrial respiratory activity was measured by the rate of resazurin reduction. **F)**.Tumorigenicity was measured by Matrigel invasion assay.

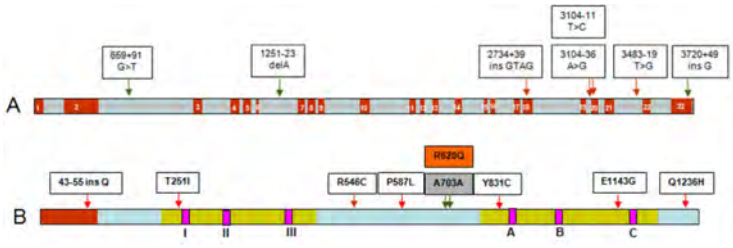

Figure 2. POLG mutations in breast tumors and breast cancer cell lines

A) Intron/Splice variants in the POLG genome;**B**) Mutations in the POLG protein with amino acid change. Green and red arrows indicate the novel variants and disease-associated mutations/polymorphisms, respectively. The grey and orange boxes indicate the novel silent and missense mutations, respectively; **C**) The amino acid conservation at the mutant residue of R628Q, a novel missense mutation observed in the linker region.

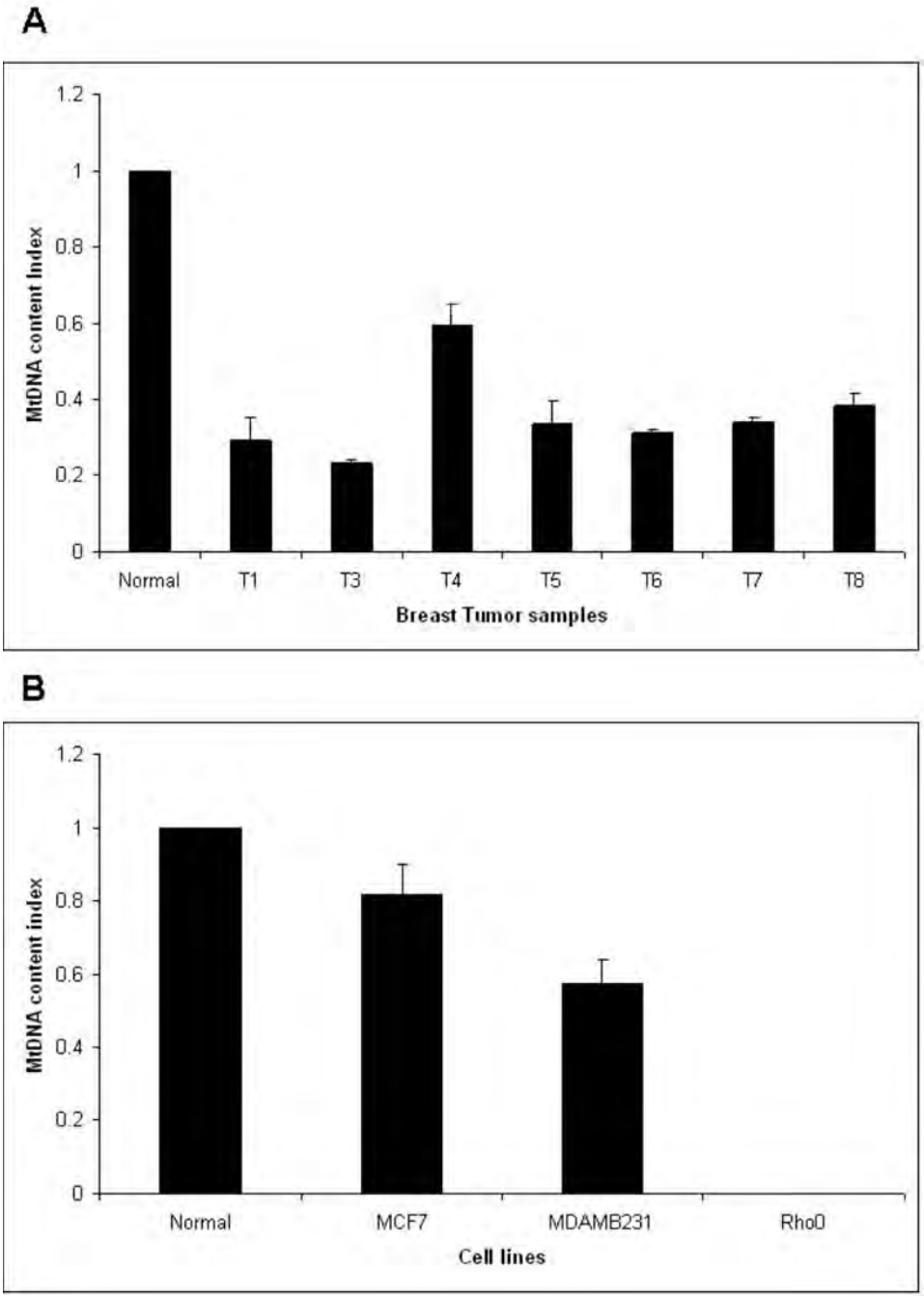

Figure 3. Decreased mtDNA content

A) in breast tumor samples and **B**) in breast cancer cell lines. The ratio of mtDNA to nuclear DNA was used as an index for measuring the mtDNA content (described in material methods).

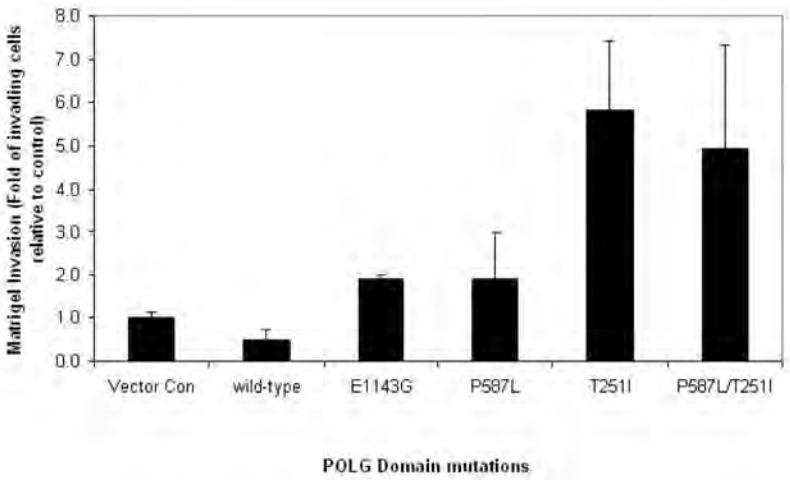

Figure 4. Breast tumor POLG mutations lead to increased tumorigenicity

Matrigel invasion of MCF7 Tet-on Advanced cells expressing representative mutations in POLG identified in primary breast tumors. Cell were treated with 1000 ng/ml doxycycline and sorted for GFP fluorescence. Cells were grown in the presence of doxycycline for 5 day and the Matrigel invasion was carried out. Data represents mean percentage of invading cells normalized to negative vector control ± 1 SEM.

Table 1

POLG mutations in breast tumors. The table lists POLG mutations identified in primary breast tumor tissues (19) and breast cancer cell lines. The heterozygous mutations are marked by single asterisk and homozygous mutations are marked by double asterisks.

	Number of Tumors	Mutant Percentage	POLG1 mutations															
Breast Tumor samples	2	10.5		752 C>T *		1780C>T *					2738D>T*	559+9T G>T *		2734+39 insGTAG**	3104-3E A>G *	3104-11 T>C*	3483-10 T>G*	3720+46 insG *
	2	10.5	ins.CAG 127-156 *									559+9T G>T *		2734+39 insGTAG*	3104-3E A>G *	3104-11 T>C*	3483-10 T>G*	3720+46 insG *
	1	5.3									3738G>T*	559+9T G>T*		2734+39 insGTAG*	3104-3E A>G *	3104-11 T>C*	3483-10 T>G*	3720+46 insG *
	1	5.3											1261-23delA *					
	1	5.3	ins.CAG 127-156 *											2734+39 insGTAG*				
	1	5.3			1838 C>T *		1883 G>A *	2188 C>A *				559+9T G>T *		2734+39 insGTAG*			3483-10 T>G*	
	1	5.3									3708(6)T*	559+9T G>T **		2734+39 insGTAG**	3104-3E A>G**	3104-11 T>C**		3720+46 insG **
	1	5.3	ins.CAG 127-156 *							3428A>G *		559+9T G>T *		2734+39 insGTAG**	3104-3E A>G *	3104-11 T>C*	3483-10 T>G*	3720+46 insG *
Breast Cancer cell lines	1	50										559+9T G>T *		2734+39 insGTAG*	3104-3E A>G *	3104-11 T>C*	3483-10 T>G*	3720+46 insG *
	1	50							2402 A>G **									
Genomic region			Exon 2	Exon 3	Exon 9	Exon 16	Exon 16	Exon 12	Exon 18	Exon 21	Exon 23	Intron 2	Intron 6	Intron 9	Intron 16	Intron 16	Intron 21	3'UTR
Protein domain				Exonuclease	Linker	Linker	Linker	Linker	Polymerase	Polymerase								
Amino acid change			ins Gln-43-Ile	Thr251Ile	Arg549Cys	Pro587Leu	Arg628Cys	Ala760Ala	Tyr831Cys	Glu1143Gly	Gln1236His							
Disease association /Significance			Tissue specific cancer, Parkinson's disease	PEO, Infantile hepatocerebral syndrome	Polymorphism	PEO, Harvill hypomyelination syndrome	Norm. missense	Novel, silent	PEO & Parkinsonism	Ataxia Neuropathy	Polymorphism	Norm. intronic	Norm. synmic	Polymorphism	Polymorphism	Polymorphism	Polymorphism	Novel intronic

Table 2

Mitochondrial DNA mutations in breast tumors. Complete mtDNA was sequenced in representative samples (n4), containing POLG mutations T251I (Exonuclease domain) and P587L (Linker domain); ins Gln 43-55 and intronic variants.

Variant	Mutant percentage (n=4)	Region	Amino Acid Change	Association with Cancer and other diseases
A93G	50	D-loop	N/A	Colorectal and Gastric tumors
T152C	50	D-loop	N/A	Ovarian/ Squamous cell carcinoma
A263G	100	D-loop	N/A	Oral Cancer
C309CC	100	D-loop	N/A	Multiple tumors
C315CC	100	D-loop	N/A	Multiple tumors
C16169T	50	D-loop	N/A	Polymorphism
T16172C	50	D-loop	N/A	MNGIE/Oral Cancer
C16261T	50	D-loop	N/A	Oral Cancer
T16311C	50	D-loop	N/A	Oral Cancer
C16320T	50	D-loop	N/A	Oral Cancer
A750G	100	12S rRNA	N/A	Polymorphism
A1438G	100	12S rRNA	N/A	Polymorphism
A2706G	50	16S rRNA	N/A	Oral Cancer
A4769G	100	ND2	syn	Polymorphism
G6755A	50	COI	syn	Polymorphism
A8860G	100	ATPase 6	T-A	Polymorphism
A8869G	50	ATPase 6	M-V	Polymorphism
G13759A	50	ND5	A-T	Polymorphism
T15214C	50	CYT B	syn	Polymorphism
A15326G	100	CYT B	T-A	Polymorphism